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# The Human Growth Hormone Gene Is Regulated by a Multicomponent Locus Control Region

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**The five-member human growth hormone (*hGH*)/chorionic somatomammotropin (*hCS*) gene cluster encodes the pituitary-specific *hGH-N* gene and four highly related genes (*hGH-V*, *hCS-A*, *hCS-B*, and *hCS-L*) that are expressed only in the placenta. When the *hGH-N* or *hCS-A* gene, together with all previously identified *cis*-acting regulatory sequences, was integrated into the mouse genome, it was expressed only sporadically and at low levels in the transgenic target organs. DNase I mapping of chromatin from expressing and nonexpressing cell types was used to identify a pituitary-specific set of DNase I-hypersensitive sites (HS) and a set of HS common to both the pituitary and placenta, centered approximately 15 and 30 kb 5′ of *hGH-N*, respectively. When contained on a cosmid insert in their native genomic configuration, these HS consistently directed high-level, pituitary-specific expression of *hGH-N* in transgenic mice and appeared to define a locus control region required for *hGH-N* expression. Individually, each set of HS was able to mediate position-independent *hGH-N* expression in the pituitary but demonstrated loss of physiologic control and loss of tissue specificity. The gene-proximal set of HS contained a potent enhancer activity in the pituitary, while the more distal set appeared to function primarily to establish site-of-integration independence. These data indicate that synergistic interactions among multiple elements are required to restrict *hGH-N* transcription to the pituitary and generate appropriate levels of expression. In addition, these results suggest a role for both shared and unique regulatory sequences in locus control region-mediated expression of the *hGH*/*hCS* gene cluster in the pituitary and possibly the placenta.**

Growth hormone (GH) is the prototypic member of the cytokine superfamily that includes interleukins, growth factors, cytokines, leukemic inhibitory factor, and neurotrophic factors (16, 57). Most species contain a single *GH* gene, whereas in humans, the *GH* gene has expanded by repeated duplications into a five-gene cluster spanning 48 kb (3, 10). Although these five genes and their respective promoters have greater than 90% sequence identity (10), they demonstrate high-level, mutually exclusive expression in the pituitary and placenta. Expression of human *GH-N* (*hGH-N*), the most 5′ gene in the cluster, is limited to the somatotrope and somatolactotrope cells of the anterior pituitary and comprises 3% of the total pituitary mRNA (10, 39). In contrast, transcription of the other four genes is restricted to the syncytiotrophoblast layer of the placental villi (44, 47), and transcripts from two of these human chorionic somatomammotropin (*hCS*) genes, *hCS-A* and *hCS-B*, contribute 3.5% to the total placental mRNA (10, 65).

*cis*-acting regulatory elements adjacent to both *hGH-N* and *hCS*, defined in cell transfection assays, are unable to consistently direct appropriate expression in transgenic mice. Transcription of the transfected *hGH-N* in rat pituitary GH3 or GC cells is dependent on promoter-proximal elements that include two binding sites for the pituitary-specific factor pit-1/GHF-1 (5, 9, 34, 54) and a single site for Zn15 (41). However, the placentally expressed *hGH-V* and *hCS* genes have proximal promoters that are highly similar to *hGH-N* and function nearly as well as the *hGH-N* promoter in rat pituitary cells (49). Pituitary-specific repressor sequences located 2 kb 5′ of all four placentally expressed genes (48) and placenta-specific enhancer sequences located 3′ of the *hCS* genes (65) may serve to suppress pituitary expression in vivo and limit transcription to the placenta. When tested for expression in mice, *hGH-N* directed by its proximal promoter or by an additional 4.6 kb of 5′ and 26 kb of 3′-flanking sequences is either not expressed or only poorly expressed in transgenic pituitaries (30, 51). Likewise, an *hCS-A* transgene accompanied by 5.4 kb of 5′ and 7.2 kb of 3′ DNA, including all defined control elements, is shown here to be poorly expressed in the transgenic placenta. These data suggest that the presently recognized proximal promoter elements of the genes in the *hGH-N*/*hCS* gene cluster are insufficient to establish appropriate expression in vivo.

The majority of the genome is packaged into a largely inaccessible chromatin conformation. This higher-order chromatin structure is locally disrupted in the vicinity of genes that are actively transcribed or poised for transcription (reviewed by Gross and Garrard [27] and Wallrath et al. [66]). Many promoter and enhancer sequences, capable of driving tissue-specific, high-level expression in cell transfection systems, are subject to site-of-integration or position effects after integration into the genome of transgenic mice. These effects reflect the influence of neighboring chromatin on the integrated transgene and are evidenced by the failure of the transgene to be reproducibly and appropriately expressed (29, 35, 51).

The remodeling of chromatin structure necessary for regulated gene expression appears to be an active process, governed by the interaction of tissue-specific and general *trans*-acting factors with *cis*-acting regulatory sequences (reviewed by Winston and Carlson [67] and Peterson and Tamkun [52]).

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Genetic evidence demonstrates that *cis*-acting sequences remote from a gene promoter are critical mediators of tissue-specific changes in chromatin structure that presage the activation of promoter-proximal elements (33, 60, 63). For example, certain limited deletions far upstream of the β-globin gene cluster completely inactivate expression of all genes in the cluster (reviewed by Townes and Behringer [62] and Dillon and Grosveld [17]). The chromatin domain encompassing this inactivated β-globin gene cluster is DNase I resistant and late replicating. In contrast, the normal β-globin gene cluster in erythroid cells replicates early in the cell cycle and resides in an open, DNase I-sensitive chromatin domain (22). These data suggest that β-globin expression is mediated by upstream regulatory elements that function to establish an open chromatin domain essential for subsequent transcriptional activation. Because of their crucial gene activation function in the β-globin gene cluster, such dominant regulatory elements have been termed locus control regions (LCRs) (23, 28, 50, 64). LCRs may be one of a class of *cis* elements that function to alter chromatin structure and activate or silence gene expression.

The regulation of gene expression via modulation of chromatin domains is not limited to the β-globin gene cluster. A multiplicity of *cis* elements that may work in conjunction with LCRs or independently have been described and include insulator elements (14), boundary elements (19), specialized chromatin structures (37), and facilitators (2). Additional major regulatory elements marked by tissue-specific DNase I-hypersensitive sites (HS) and functioning at the level of chromatin structure have also been identified (e.g., human α-globin −40HS [31] and the upstream regulatory element of the CD34 locus [45]). These elements may establish the functional boundaries for segments of activated chromatin or increase the efficiency of classical enhancer elements in a chromatin environment. For example, the chicken lysozyme gene is flanked by boundary elements that separate a domain of generalized DNase I sensitivity from surrounding potentially inactive chromatin. A combination of boundary elements and matrix attachment sites with additional *cis* elements functions to alter chromatin and mediates lysozyme gene activation (6, 7, 32, 58). Chromatin remodeling elements can also function as developmental activators as is seen in the control of the homeotic genes in the developing *Drosophila* embryo (reviewed by Kennison [38] and Schaffer et al. [56]) and as developmental silencers as observed in the control of the yeast mating-type genes (11, 42; reviewed by Peterson and Tamkun [52]). Thus, chromatin remodeling is an ancient and ubiquitous means of regulating gene expression.

In this study, a set of regulatory elements far upstream from the *GH* gene cluster is identified. These elements colocalize with a set of tissue-specific HS in the native chromatin of cells expressing genes in the cluster. Functional analyses of these regulatory elements in transgenic mice indicate that the individual components of this regulatory region overcome chromatin-mediated position effects and contribute to native levels of gene expression in the pituitary. When these elements were deleted or tested in isolation, loss of tissue specificity was observed. These elements are hypothesized to represent an LCR for the *GH* gene.

## MATERIALS AND METHODS

**Cosmid cloning and mapping.** Cosmid GH5 was generously provided by K. H. Choo (13). Cosmids 11B1, K2B, and PAJ were isolated from human genomic libraries constructed in the pWE15 vector (Stratagene library 951202). Cosmids K2B and PAJ were isolated by hybridization to a 520-bp *Nar*I-*Xma*I fragment of the *hGH-N* gene. Cosmid 11B1 was isolated by using a *Sma*I restriction fragment from the GH5 cosmid spanning kb −32.7 to −31.3. Mapping was performed by indirect end labeling of partial digestions of the cosmid inserts as suggested by Stratagene.

**Isolation of intact nuclei. (i) Placenta.** A procedure was developed to isolate nuclei selectively from the syncytiotrophoblast cells of human term placentas. Villous fragments were washed in ice-cold Hanks buffered saline solution, minced finely, and suspended in Hanks buffered saline solution. Fragments passing through a 10-gauge screen were pelleted and resuspended in 40 ml of 150 mM $NH_4Cl$ containing 0.5 g of $NH_4HCO_3$ per 10 g of tissue. This procedure causes osmotic lysis of the syncytiotrophoblast cells as a result of their known high levels of carbonic anhydrase (1). After 30 to 40 min at 4°C, the preparation was pelleted at 1,000 × *g*, and the pellet was resuspended in 40 ml of buffer A (15 mM Tris [pH 7.4], 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) containing 1 M sucrose and centrifuged at 300 × *g* for 10 min at 4°C; the supernatant was collected and recentrifuged at 4,300 × *g* for 15 min at 4°C. The pellet was resuspended in 10 ml of RB buffer (26) and centrifuged for 7 min at 1,000 × *g*. The upper white phase was resuspended in 1 ml of RB buffer and examined under the microscope to confirm the presence of intact nuclei. We typically obtained 1.7 mg of nuclei from 10 g of tissue in $NH_4Cl$-$NH_4CO_3$, and the $A_{260}/A_{280}$ ratio was 1.5.

**(ii) Pituitary.** An hGH-secreting pituitary adenoma was obtained immediately following surgery. Mouse pituitaries were isolated after decapitation. Pituitary cells were dissociated in cell-free dissociation buffer (Boehringer Mannheim). Nuclei from the dissociated pituitaries and from K562 cells were isolated as previously described (26).

**DNase I mapping of intact nuclei.** Nuclei were resuspended at 1 mg (20 $A_{260}$ units)/ml in RB buffer, and a baseline (0-min) aliquot was withdrawn. RB buffer containing 3 mM $CaCl_2$ and 3 μg of DNase I (Gibco-BRL) per ml was added to yield final concentrations of 670 μg of nuclei per ml, 1 μg of DNase I per ml, and 1 mM $CaCl_2$. The sample was incubated at 37°C. Aliquots were withdrawn at 2, 4, 8, and 15 min, and each reaction was terminated by adding EDTA to 50 mM followed by digestion of the nuclei with 120 μg of proteinase K per ml in 0.8 M NaCl–0.5% sodium dodecyl sulfate, extraction with phenol and chloroform, and then ethanol precipitation. DNase I-digested DNA samples were subsequently digested with restriction enzymes and transferred to Zetabind nylon membranes for hybridization.

**Construction of *hGH-N* transgenes.** *−22.5hGH* was generated by truncation of *−40hGH* at the *Cla*I site. *−0.5hGH* is the native 2.6-kb *Eco*RI fragment. *HSIII*, *VhGH* was generated by ligation of the 5′-terminal 11.5-kb *Cla*I fragment of the cosmid K2B insert to *−0.5hGH* in the native orientation.

**Isolation of *hCS-A*.** Total human genomic DNA was digested to completion with *Hin*dIII and fractionated on a 0.8% agarose gel. A gel slice corresponding to the 15-kb fraction was excised, and the DNA was isolated on glass beads (Qiagen). A phage library was constructed with this fraction in λDash II (Stratagene) and was screened with an *hCS* cDNA probe by standard techniques. The *hCS-A*-containing insert was released by *Not*I digestion, subcloned into pBluescript II+ (Stratagene), and used as transgene construct *15kbhCS-A*.

**Generation and analysis of transgenic mice.** Inserts were released from vector sequences by restriction digestion, separated on agarose gels, isolated with glass beads, purified through an Elutip (Schleicher & Schuell), and adjusted to a concentration of 2 ng/μl in 10 mM Tris (pH 7.6)–0.1 mM EDTA prior to microinjections into fertilized oocytes from superovulated (C57BL/6 × SJL)$F_1$ females mated to (C57BL/6 × SJL)$F_1$ males. Positive founders were detected by dot blot analyses of tail DNA. The integrity of the transgenes was determined by Southern blot analyses of *Bgl*II-digested tail DNA. Transgene integrity was intact in all reported lines. Two of the original *−7.5hGH* lines contained gene rearrangements and were not used or reported. The BN line (*−40hGH*) contained two integration sites. As detailed in Results, these two sites were segregated; one contained a 5′ deletion which was informative and thus studied. Transgene copy numbers were quantitated by PhosphorImager analysis of Southern blots. The *hGH* transgene signal was divided by the hybridization signal from a single-copy mouse gene, either *CRIP* (4), *CRP* (40), or *DBP* (15), on the same filter.

**RNA isolation and analysis.** Pituitary, brain, kidney, liver, placenta, and spleen RNAs were prepared from tissues isolated from mice immediately following decapitation (12). For reverse transcription (RT)-PCR analyses, 0.5 μg of pituitary RNA was used for all lines except the *HSIII,VhGH* lines, which required 2 μg; for all other tissues, 4 μg of RNA was used. The sequences of the 5′ and 3′ PCR primers were 5′-GCCTGCTCTGCCTGC-3′ (exon 2) and 5′-GACTGG ATGAGCAGCAG-3′ (exon 4), respectively. RT reactions were at 42°C in 40 μl of RT buffer and 7.5 U of avian myeloblastosis virus reverse transcriptase. After heat inactivation of the enzyme at 94°C for 5 min, PCR amplifications were carried out for 20 cycles each in a 50-μl volume of RT buffer with 5 U of Amplitaq. The temperatures for annealing, elongation, and denaturation were 45, 72, and 94°C, respectively. Samples were analyzed on 6% denaturing polyacrylamide gels and quantitated with a PhosphorImager. For the PCR standard curve (Fig. 6A), input concentration ratios of linearized *hGH* to mouse *GH* (*mGH*) cDNAs were 50 pg/5 ng, 500 pg/5 ng, 5 ng/5 ng, 5 ng/500 pg, and 5 ng/50 pg. The standard curve obtained was highly reproducible, and the average of five individual standard curves was used to calculate transgenic pituitary *hGH*/*mGH* mRNA ratios. Only the major, 170-bp *hGH* cDNA band was used for quantita-

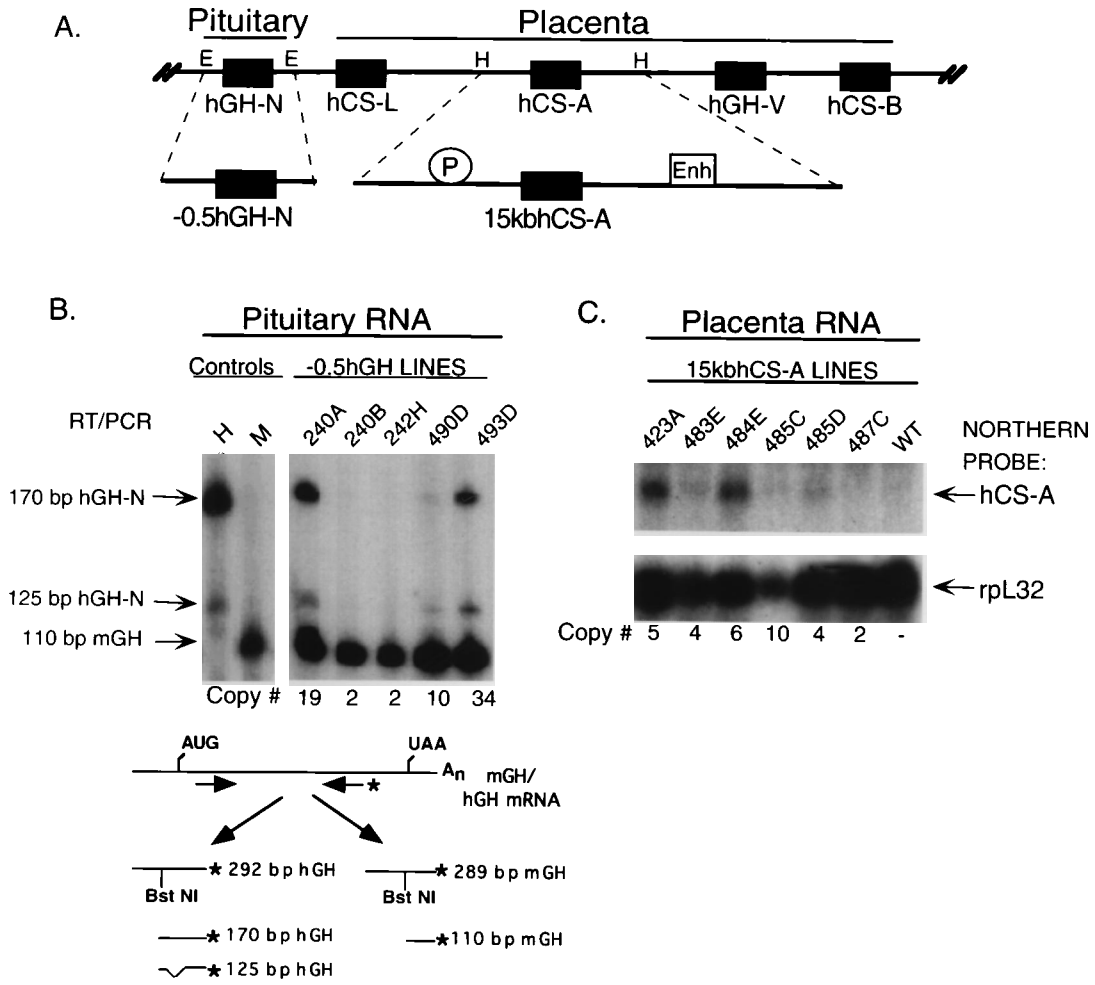


FIG. 1. Expression of minimal *hGH-N* (*−0.5hGH*) and *hCS-A* (*15kbhCS-A*) transgenes is subject to chromatin position effects. (A) Diagram of the *hGH* cluster and the *−0.5hGH* and *15kbhCS-A* transgenes. The arrangement of the five genes in the *hGH* cluster is shown on the first line (3, 10). The filled rectangles represent each of the five genes. The site of native expression, pituitary or placenta, is noted above each gene. The *Eco*RI (E) and *Hin*dIII (H) sites delimiting the *−0.5hGH* and the *15kbhCS-A* transgenes, respectively, are shown. The two regions of the cluster isolated and injected into mouse oocytes (*−0.5hGH* and *15kbhCS-A*) are shown below the cluster. The previously identified 5′ and 3′ control elements that contribute to appropriate *hCS-A* expression, P element (P) and enhancer (Enh), are indicated. (B) Analyses of *GH* mRNA in the pituitaries of *−0.5hGH* transgenic mice. An autoradiograph of the RT-PCR products is shown. The individual transgenic line designations and corresponding transgene copy numbers are noted above and below each lane, respectively. The two control lanes represent analyses of human *GH* mRNA present in a stably transfected cell line expressing *hGH-N* (H) (21) and *mGH* mRNA (M) from wild-type mouse pituitaries. The positions of the full-length and alternatively spliced *hGH-N* mRNAs (21) and of *mGH* mRNA products are indicated by the arrows at the left. A diagram of the RT-PCR assay is shown below the autoradiograph. The first line represents the 77% identical *hGH* and *mGH* mRNAs; the initiation codon (AUG), termination codon (UAG), poly(A) tail ($A_n$), and primers used for RT and PCR (arrows) are indicated. Primers correspond to regions of structural identity between *hGH* and *mGH* mRNAs. The 3′ primer was $^{32}$P-labeled (*). The second line shows the cDNA segments generated by RT-PCR of *hGH* mRNA (left) and *mGH* mRNA (right) and the locations of the *Bst*NI sites used in the analysis. The end-labeled cDNA fragments generated by *Bst*NI digestion are shown at the bottom, with the size and identity of each fragment noted. Quantitation of the relative levels of *hGH-N* and *mGH* mRNAs in pituitary samples was based on the relative intensities of the 170- and 110-bp fragments (see Materials and Methods and Fig. 6A). (C) Northern blot analysis of placental mRNA from *15kbhCS-A* transgenic fetuses. The line designations and corresponding transgene copy numbers are shown above and below each lane, respectively. Each fetus was the product of a mating of a male hemizygote with a wild-type female. The transgenic status of the fetus was determined by DNA analysis of the body; RNA was isolated from the corresponding placenta. The Northern transfer was hybridized sequentially with an *hCS-A* cDNA probe and a cDNA probe corresponding to mouse ribosomal protein L32 (rpL32).

tion of *hGH* mRNA concentration because a 125-bp band that comigrated with the minor alternative splicing product of *hGH-N* (21) was occasionally obtained as a background band with *mGH* mRNA alone. All pituitary RNA samples were assayed in three or more individual experiments, and determinations were typically performed on multiple pituitary RNA samples for each line.

Northern (RNA) analyses of 1 to 5 μg of pituitary RNA or 10 μg of other RNAs were carried out on 1.5% agarose gels containing formaldehyde in morpholinepropanesulfonic acid (MOPS) (55) and then transferred to GeneScreen Plus. PhosphorImager quantitation of a subsequent hybridization with ribosomal protein L32 cDNA (46) was used to normalize for loading differences.

**Growth curves.** Pups from litters generated by mating a hemizygote of each line with a wild-type female were weighed at weekly intervals for 13 weeks. Transgenic status was determined by tail blot after the completion of the study.

## RESULTS

**Promoter-proximal transcription control elements of *hGH-N* and *hCS-A* are insufficient to establish reproducible and appropriate levels of expression in vivo.** Previous studies have suggested that the proximal promoter elements of *hGH-N* lack sufficient information for appropriate expression in transgenic mice (30, 51). To confirm and extend these studies, we generated a series of transgenic lines carrying the individual *hGH-N* and *hCS-A* genes, the two most highly expressed genes in the *hGH* cluster (Fig. 1A). A 2.6-kb genomic *Eco*RI restriction

fragment encompassing the entire *hGH-N* gene along with 500 bp of 5′-flanking sequences (*−0.5hGH*) was isolated and used to generate transgenic mice. Five independent *−0.5hGH* founders, each of which was fertile and able to establish a line, were generated. A 15-kb *Hin*dIII genomic fragment containing the *hCS-A* gene along with 5.4 kb of 5′-flanking region and 7.2 kb of 3′-flanking region (*15kbhCS-A*), containing both the 5′ P element (48) and the 3′ enhancer (65) thought to be important in tissue specificity and high-level expression, respectively, was cloned and used to generate six transgenic founders, each of which established a line. Transgene copy number and integrity for each line was established by Southern analysis of tail DNA from an $F_1$ or $F_2$ animal hemizygous for the transgene.

GH production is subject to both positive and negative feedback at the levels of transcription and secretion (reviewed by Theill and Karin [61] and Lewis [39]). Overexpression of GH has the potential to trigger a negative feedback loop that depresses transcription of *GH* genes (mouse or human). For this reason, the level of *hGH* transgene expression was measured in the pituitaries of individual $F_1$ transgenic mice and was compared with the levels of endogenous *mGH* mRNA in an RT-PCR assay. This assay generates distinct cDNA fragments corresponding to *hGH* and *mGH* mRNAs (Fig. 1B; see also Fig. 6A).

Analysis of pituitary RNA in the *hGH-N* transgenic mice revealed expression in only three of the five lines (Fig. 1B). Comparison with the *mGH* mRNA levels indicated very low levels of transgene expression in two of the expressing lines (490D and 493D), while the level of *hGH* mRNA in a third line (240A) was closer to that of *mGH* mRNA. Variable and sporadic transgene expression was also observed in placentas of fetuses from six lines bearing the *15kbhCS-A* transgene (Fig. 1C). In this case, since there is no comparably expressed mouse placental mRNA that might cross-hybridize, analysis was carried out by Northern blotting. Among the six lines, two (423A, and 484E) showed unequivocal *hCS-A* mRNA signals, two (483E and 486D) showed trace levels, and two (485C and 487C) appeared to be negative. Thus, proximal regulatory elements appeared to be insufficient to drive either the *hGH-N* or *hCS-A* expression in their respective target tissues in a reproducible fashion and at wild-type levels.

**Distinct patterns of DNase I HS 5′ to the *hGH* cluster in nuclei isolated from pituitary and placentas.** The sporadic expression of the isolated *hGH-N* and *hCS-A* transgenes suggested that additional regulatory elements required for reproducible, high-level expression were lacking. To search for these elements, DNase I mapping was carried out on chromatin from human pituitary and placental tissue. To facilitate these mapping studies, we isolated overlapping cosmid clones that extended the cloned region 60 kb 5′ to the cluster and 40 kb 3′ (Fig. 2A). Cosmids K2B and PAJ encompass *hGH-N* and extend to kb −40 and −22.9, respectively. Cosmid GH5 extends from kb −34 to +4 (13), and an overlapping cosmid, 11B1, extends to kb −63. Cosmid 20B extends 3′ to the cluster. A restriction map of each cosmid insert was established and demonstrated to be colinear with the respective region of the human genome (not shown).

Nuclei isolated from an acromegalic pituitary adenoma oversecreting hGH-N and from the normal hCS-secreting syncytiotrophoblast cells of term placentas were subjected to limited DNase I digestion, then complete digestion with either *Eco*RI or *Hin*dIII, and Southern blot analysis (Fig. 2B). DNase I HS were mapped by indirect end labeling using probe P1, P2, or P3 (Fig. 2A). Analysis of the *Eco*RI fragment spanning sequences 44.5 to 21.1 kb 5′ of *hGH-N* revealed two DNase I HS at coordinates −32.5 and −27.5 that were common to placenta and pituitary and a third placenta-specific HS at coordinate −30.5. Two additional HS specific to the pituitary at kb −14.6 and −15.4 were detected by analysis of an overlapping *Hin*dIII fragment (Fig. 2C). Rehybridization of the filter shown in Fig. 2C with probe P3 confirmed the positions of these two additional sites (not shown). These data demonstrated shared as well as unique DNase I HS in the chromatin of primary pituitary and syncytiotrophoblast cells. The region 3′ to the gene cluster was similarly mapped, and a single HS was identified at coordinate +76 in placental DNA. The presence of this site in the pituitary could not be tested because of the paucity of the human pituitary adenoma tissue. The five HS mapped 5′ to the *hGH* gene cluster in pituitary and placental nuclei were assigned numbers I through V. K562 human erythroid cell nuclei were similarly analyzed and lacked any HS in this region (not shown).

DNase I HS more proximal to *hGH-N* were mapped using a *Hin*dIII fragment spanning kb −7.2 through +16.5. As expected, the *hGH-N* promoter was DNase I sensitive in pituitary nuclei (not shown). Placenta-specific HS flanking *hGH-N* were detected and might point to repressors of *hGH-N* expression in this tissue (Fig. 2D). The adjacent region from kb −9.2 to −7.2 was mapped in placental nuclei, and no additional HS sites were identified.

A summary of DNase I HS mapping (Fig. 2E) demonstrated a tissue-specific pattern of HS with both a pituitary-specific set of HS (HS I and II), a placental-specific HS (IV), and a subset of HS common to the two expressing tissues, pituitary and placenta (HS III and V).

***hGH-N* with 40 kb of 5′-flanking region overcomes the site-of-integration effects.** The identification of five DNase I HS located 5′ of the cluster suggested the presence of corresponding control elements that might be essential for establishing appropriate transcriptional activation. To test this, the K2B cosmid insert (Fig. 2A and 3A), containing *hGH-N* and 40 kb of contiguous 5′-flanking DNA, was introduced into the mouse genome and tested for expression. Five lines carrying this *−40hGH* transgene were generated. One of the *−40hGH* founders, BN, contained two independent transgene integration sites that were segregated by outbreeding to generate two independent mouse sublines. The BN2 subline contained three head-to-tail copies of full-length *−40hGH*, while BN31 contained a single 5′-terminally-deleted copy of the *hGH-N* gene that was accompanied by 5 kb of 5′-flanking sequence (*−5.0hGH*). *hGH* transgene expression was measured in the pituitaries of individual $F_1$ transgenic mice relative to the levels of endogenous *mGH* mRNA. In contrast to the marked inconsistency in *−0.5hGH* transgene expression (Fig. 1B), all five *−40hGH* lines expressed *hGH-N* (Fig. 3B). Furthermore, the levels of *hGH-N* mRNA in each of the *−40hGH* lines appeared comparable or greater than endogenous *mGH* mRNA. The BN31 line (*−5.0hGH*) lacking the 5′ sequences encompassing HS I through HS V did not express the transgene (Fig. 3B). Thus, expression of the *hGH-N* transgenes containing 5 kb or less of 5′-flanking sequences was subject to site-of-integration effects, while sequence elements located between kb −40 and −5.0 conferred site-of-integration independent, high-level expression on *hGH-N* in the transgenic pituitary.

**Reestablishment of HS in the transgenic mouse pituitary.** The consistent, high-level expression of the *−40hGH* transgene compared with the *−0.5hGH* transgene indicated the existence of functionally important control elements in the distal 5′-flanking region of the *hGH-N*. If these elements were coincident with HS mapped in the human pituitary chromatin, they should be reestablished in the transgene in transgenic pituitary chromatin. Pituitaries from 50 transgenic adults car-

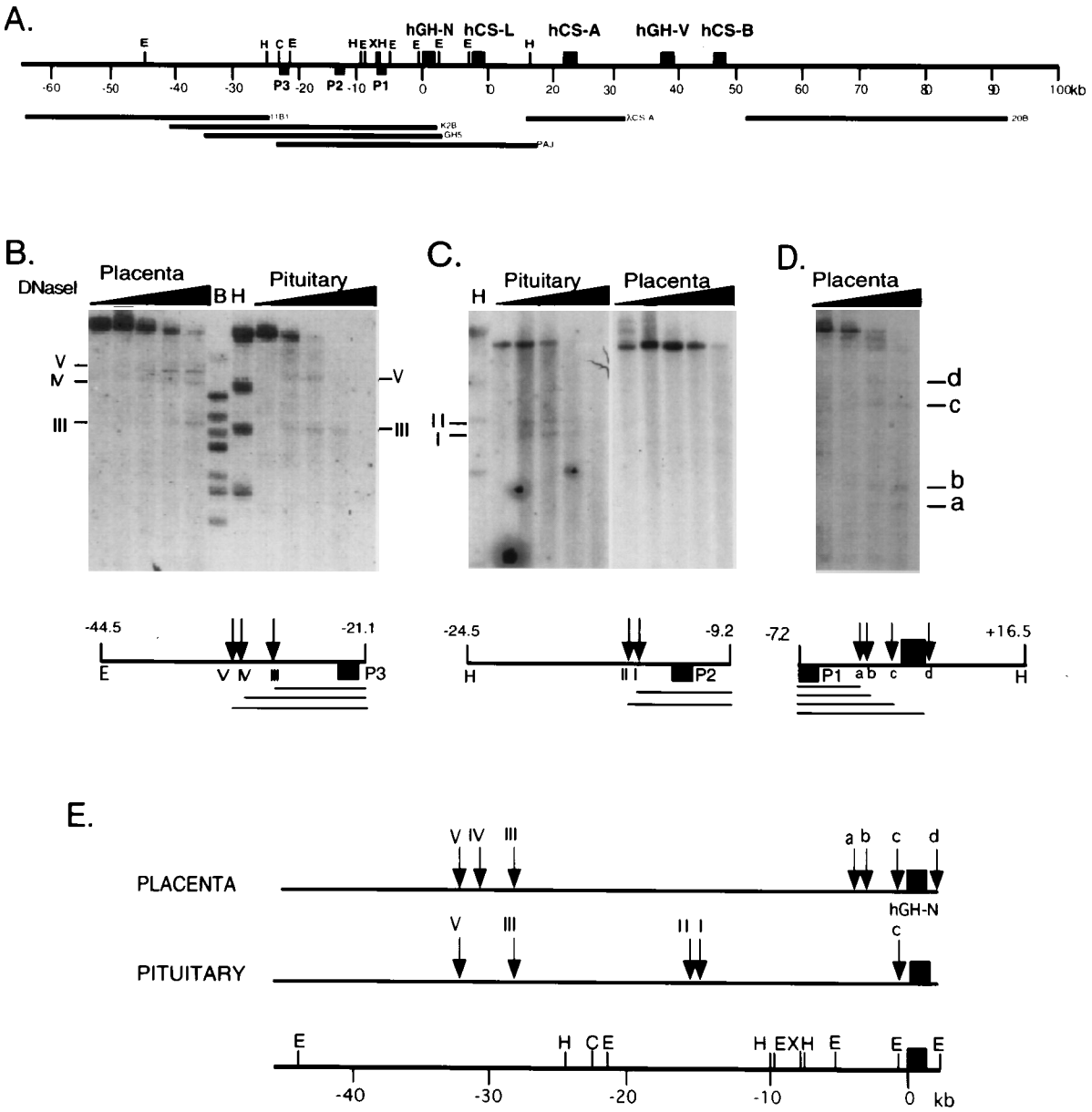


FIG. 2. DNase I HS flank the *hGH* cluster. (A) The *hGH* cluster and cosmid clones extending into the 5′- and 3′-flanking regions. The arrangement of the five genes in the *hGH* cluster is as in Fig. 1A. Coordinates are shown below the map; +1 is the start site of *hGH-N* transcription. Four cosmid clones encompassing 63 kb of 5′-flanking sequences adjacent to the *hGH* cluster, a phage clone encompassing the *hCS-A* and surrounding control elements (see Fig. 1A), and a cosmid extending for 40 kb 3′ to the cluster are illustrated and labeled. The restriction map is limited to sites in the 5′-flanking regions that are relevant to subsequent studies: *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), and *Xho*I (X). The positions of the three probes used for DNase I mapping studies are indicated by the three filled rectangles below the map (P1, P2, and P3). (B to D) Identification of DNase I HS 5′ of the *hGH-N* cluster. Nuclei isolated from a human pituitary GH-secreting adenoma (pituitary) and human term placental syncytiotrophoblasts (placenta) were digested with DNase I for increasing periods of time (wedges). DNA was then isolated, digested with restriction enzymes, and analyzed by Southern blotting using indirect end labeling with P1, P2, or P3 as indicated. Representative autoradiograms are shown. The diagram below each autoradiogram illustrates the restriction fragment being analyzed and the identity and position of each hybridization probe. The positions of the major subbands generated by DNase I in placenta and pituitary are indicated along the sides of the autoradiograms. The position of each DNase I HS site relative to the end of the restriction fragment is noted in the diagram below each panel by both a vertical arrow and a line representing the length of the subband. (B) DNase I mapping of placental and pituitary chromatin extending from 44.5 to 21.1 kb 5′ of *hGH-N*. The two center lanes contain $^{32}$P-labeled *Bst*EII (B) and *Hin*dIII (H) restriction fragments of λDNA as size markers. (C) DNase I mapping of pituitary and placental chromatin extending from kb −24.5 to −9.2. (D) DNase I mapping of placental chromatin between kb −7.2 and +16.5. The position of *hGH-N* is designated by the gray box above the line. Two additional HS detected in this study were out of the linear range of the gel for sizing and were not assigned letters or included on the map. (E) Summary of DNase I HS 5′ to the *hGH* cluster. The *hGH-N* gene is shown as a gray rectangle at the right of the map. HS are indicated by vertical arrows; HS proximal to the *hGH-N* transcriptional start site are shown and lettered for the placenta. The remote HS are numbered in a 3′-to-5′ direction according to convention (50). Relevant restriction sites and coordinates are indicated on the bottom line.

rying the *−40hGH* transgene (line 146C) were isolated and pooled, nuclei were isolated, and DNase I HS were mapped. HS I and HS II were detected in the analysis of an *Eco*RI fragment spanning kb −21.1 to −8.7 (Fig. 3C). Analysis of a more 5′ *Eco*RI fragment, kb −41.4 to −21.1, indicated that HS III was also reestablished (Fig. 3C). HS V could not be visualized in this study because of the presence of a comigrating transgene junction fragment. Examination of a *Hin*dIII frag-

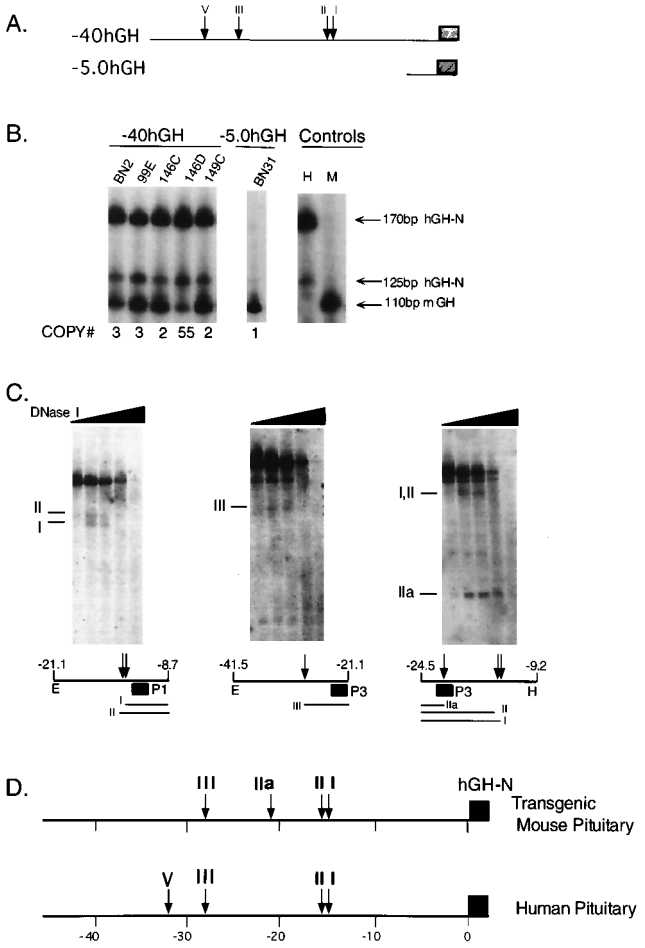


FIG. 3. *−40hGH* is expressed in a consistent manner and is associated with re-formation of the HS in transgenic mouse pituitaries. (A) Map of the *−40hGH* transgene. The first line shows the *−40hGH* transgene and the positions of the four pituitary-expressed HS. The second line shows the extent of a spontaneous deletion detected, segregated, and mapped (*−5.0hGH*) from one of the *−40hGH* lines, BN31. (B) RT-PCR analysis of pituitary mRNA from 5 independent *−40hGH* lines, the *−5.0hGH* subline, and control mRNAs. Designations are as in Fig. 1B. (C) DNase I HS re-form in the *−40hGH* transgene in pituitary chromatin of transgenic mice. DNA purified from DNase I-treated transgenic mouse pituitary nuclei (*−40hGH*, line BN2) was analyzed by restriction digestions followed by indirect end labeling of Southern blots. The regions analyzed, their coordinates, restriction sites, and locations of the probes (P1, P2, and P3) are diagrammed below each autoradiograph. (D) Summary of DNase I HS identified in *−40hGH* transgenic mouse and human pituitary chromatin. *hGH-N* is shown as a gray box, and HS are depicted by vertical arrows. The existence of HS V could not be determined in the transgenic pituitary (see text).

ment spanning kb −24.5 to −9.2 verified the re-formation of HS I and HS II and revealed an additional site at kb −22 (Fig. 3C). The latter site, referred to as HS IIa, was not detected in the hGH-secreting pituitary adenoma and may represent a factor binding site in non-hGH-N-expressing cells of the intact mouse anterior pituitary. Overall, these results, summarized in Fig. 3D, demonstrated that similar complements of HS were formed in the *−40hGH-N* 5′-flanking sequences in the transgenic mouse pituitary and in human pituitary nuclei, suggesting that these HS were associated with transgene expression.

**The HS I,II and HS III,V regions can individually overcome site-of-integration effects on pituitary transgene expression.** The four HS located 5′ to the *hGH* cluster in the chromatin of human pituitary somatotropes can be conveniently divided into a proximal group containing HS I and II and a distal group containing HS III and V. HS I and II are specific to the pituitary, while HS III and V are common to both pituitary and placenta. These two sets of HS were tested separately for function. In the initial study, HS III and V were selectively deleted from *−40hGH* by cleavage at coordinate −22.5, generating transgenic construct *−22.5hGH* (Fig. 4A). All four HS were removed by a more proximal cleavage at coordinate −7.5 (*−7.5hGH*) (Fig. 4A). Four founders carrying *−22.5hGH* and five founders carrying *−7.5hGH* were generated.

Pituitary RNA was isolated from $F_1$ hemizygotes and analyzed for *hGH* and *mGH* mRNA expression (Fig. 4B). All four lines bearing *−22.5hGH* expressed *hGH-N* mRNA in the pituitary at levels comparable to or greater than the levels of *mGH* mRNA. In marked contrast, expression of *−7.5hGH* was inconsistent; the level of *hGH-N* mRNA was significant in one line (361A), present at trace levels in two lines (244C and 516E), and absent in the remaining two lines. These data suggested that HS III and V may not be essential to pituitary expression of the *hGH-N* transgene and that the region between −7.5 and −22.5, which includes HS I and II, contains one or more transcriptional control elements important in this function.

The individual contribution of DNA segments containing HS I and II and HS III and V to *hGH-N* expression was next studied by linking gene segments containing these sites directly to the poorly expressed and position-sensitive *−0.5hGH*. An 11.5-kb fragment containing HS III and V and a 1.6-kb fragment containing HS I and II were separately ligated to *−0.5hGH* (Fig. 5A). The fragments containing HS I and II were cloned in both normal (N) and reverse (R) orientations relative to *−0.5hGH*. The three resultant constructs, *HSIII,VhGH*, *HSI,IINhGH*, and *HSI,IIRhGH*, were microinjected into mouse oocytes, and 15 founders were identified: five *HSIII,VhGH*, three *HSI,IIRhGH*, and seven *HSI,IINhGH*. Whereas fertility and viability were normal in all previously described lines as well as in the *HSIII,VhGH* lines, abnormalities were detected in the *HSI,IINhGH* and *HSI,IIRhGH* lines. Two female *HSI,IIRhGH* founders had abnormally small litter sizes (three pups per pregnancy), and all seven *HSI,IINhGH*

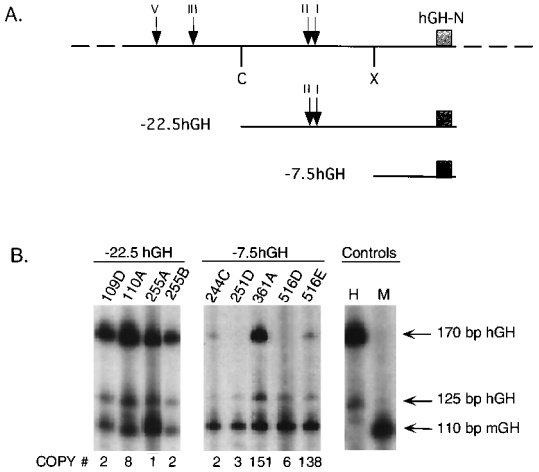


FIG. 4. Analyses of pituitary RNA from transgenic lines containing *hGH* with either 22.5 or 7.5 kb of contiguous 5′ sequences. (A) Maps of the *−22.5hGH* and the *−7.5hGH-N* transgenes. The adjacent 40 kb of 5′-flanking region containing the four pituitary HS (vertical arrows) is shown on the first line. The *Cla*I (C) and *Xho*I (X) sites used in the two truncations are indicated. Diagrams of the *−22.5hGH* and *−7.5hGH* transgenes are shown below. (B) RT-PCR analysis of pituitary RNA from four *−22.5hGH* lines and five *−7.5hGH* lines. Labeling and controls are as shown in Fig. 1B.

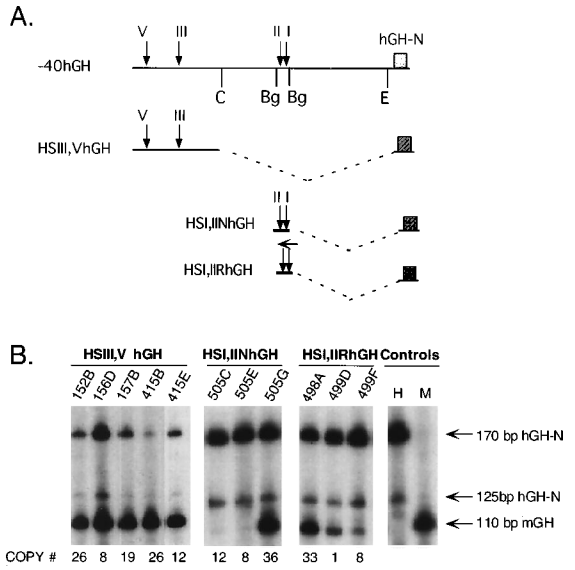


FIG. 5. Independent effects of DNA fragments containing the HS III,V or HS I,II region on *hGH* expression. (A) Generation of the *HSIII,VhGH* and *HSI,IIhGH* transgene constructs. The HS III,V and HS I,II fragments were separately ligated to *−0.5hGH-N*, the latter in both orientations. Critical restriction sites used to isolate the fragments are indicated: *Cla*I (C), *Bgl*II (B), and *Eco*RI (E). The leftward horizontal arrow indicates that the HS I,II region is present in the reverse or native orientation relative to *−0.5hGH* in the *HSI,IIhGH* transgenes. (B) RT-PCR analyses of pituitary RNA from the *HSIII,VhGH*, *HSI,IINhGH*, and *HSI,IIRhGH* lines. Labeling is as described for Fig. 1B.

transgenics were abnormal; three founders (two male and one female) were infertile and died within 3 months of birth, a fourth died at age 7 months, and the two remaining founders (505C and 505E) were infertile and thus were sacrificed for expression analysis. Only one *HSI,IINhGH* line, 505G, could be established (see below). HS I and II placed in close proximity to the *hGH-N* promoter therefore appeared deleterious to the survival of the transgenic animals.

Pituitary expression of *hGH-N* was determined in $F_1$ or subsequent progeny from each line or from the surviving founders of the *HSI,IINhGH* transgenic mice from which lines could not be established. Eleven of eleven lines or founders carrying the HS III,V or HS I,II fragment expressed *hGH-N* mRNA in their pituitaries (Fig. 5B). Transgene expression was clearly highest in the HS I,II transgenic mice. In four of these lines, the level of transgene expression substantially exceeded that of the endogenous *mGH*. The unusually high levels of transgene expression in these lines may explain the high frequency of infertility and premature death (8) observed in the *HSI,IINhGH* founders. Although all *HSIII,VhGH* lines expressed the transgene, the levels of expression of *hGH-N* mRNA were uniformly and significantly lower than in the *HSI,IIhGH* lines. These data demonstrated that DNA fragments containing either HS I and II or HS III and V can independently render the erratically expressed *−0.5hGH* site-of-integration independent in the transgenic pituitary. However, these two sets of HS differ in that the HS I,II fragment generates much higher levels of expression.

**The HS I,II region is a potent enhancer.** The level of pituitary *hGH-N* mRNA expression was determined relative to the level of endogenous *mGH* expression for all lines and normalized to the transgene copy number. These values were derived from a standard curve run in parallel with each assay which was linear over 4 orders of magnitude (Fig. 6A). The comparisons of *hGH-N* mRNA expression per transgene copy number for each of the lines are summarized in Fig. 6B. *hGH-N* was expressed in all of nine lines carrying an *hGH-N* transgene preceded by ≥22.5 kb of 5′-flanking sequences (*−40hGH* and *−22.5hGH*). The level of expression per transgene, when accompanied by 40 kb of 5′-flanking sequence, ranged from 12 to 81% of the endogenous message per transgene copy in all five *−40hGH* lines; the lowest level of expression, 12%, occurred when 55 copies of the insert were integrated. Transgene expression in the *−22.5hGH* lines was 16 to 98% of endogenous *mGH* expression; the lowest level of expression (16%) was obtained when only a single copy was integrated.

Analysis of lines bearing the *HSI,IIhGH* inserts revealed that at lower copy numbers (Fig. 6B, *HSI,IIhGH*, top four datum points), the HS I,II fragment in both orientations directed higher levels of *hGH* expression than those obtained with the intact 40-, or 22.5-kb 5′-flanking regions. These levels were also substantially higher than that of *mGH*. Because of the limited number of lines analyzed, it was not clear if the higher level of expression of *HSI,IINhGH* than of *HSI,IIRhGH* was significant. However, this possibility was supported by the high frequency of infertility and premature death observed for *HSI,IINhGH*. It should be noted that for two of the *HSI,IINhGH* founders, 505C and 505E, the level of *hGH-N* expression relative to the level of *mGH* expression was so high that it was outside the range encompassed by the standard curve. At copy numbers greater than 30, a dramatic reduction in the level of expression per copy was evident with the HS I,II fragment in both orientations (Fig. 5B, lines 505G and 498A; Fig. 6B, *HSI,IIhGH*, lowest two datum points). The expression of such high-copy-number integrations may be impeded by the creation of local heterochromatin domains (18).

Expression in the group of 16 lines lacking the HS I,II fragment (*−0.5hGH*, −5.0hGH, *−7.5hGH*, and *HSIII,VhGH*) was quite low; the maximal level was 3.5% of that of *mGH*, and 5 of 11 lines failed to express the transgene at all. Expression in the *HSIII,VhGH* lines, although reproducible, was approximately 100-fold lower than that of *−40hGH* or *−22.5hGH*. These results indicated that in addition to its ability to mediate position-independent expression in the pituitary, the HS I,II region had a potent enhancer-like activity for the *hGH-N* gene in the pituitaries of these transgenic animals.

**Tissue specificity of *hGH-N* transgene expression.** Multiple lines carrying each transgene construct were assessed for tissue-specific expression. By RT-PCR and Northern analyses of two independent *−40hGH* lines, there was no evidence of ectopic expression by the transgene in brain, kidney, liver, or spleen RNA (Fig. 7A, line 146C, and data not shown). *hGH-N* expression was largely confined to the pituitary in a *−22.5hGH* line when studied by Northern analysis (Fig. 7A, line 109D), although trace levels were detected in the spleen after prolonged exposure of the autoradiograph (not shown). Similarly, *hGH-N* expression was primarily confined to the pituitary in the *HSI,IINhGH* founders (lines 505E [Fig. 7A] and 505C [data not shown]), although trace levels were found in the brains of both lines following prolonged exposure of Northern blots. Analyses of the placentas of transgenic embryos from *−40hGH*, *−22.5hGH*, and *HSIII,VhGH* lines failed to reveal *hGH* mRNA by RT-PCR (not shown).

In contrast to the predominantly pituitary-specific expression patterns of the *−40hGH*, *−22.5hGH*, and *HSI,IINhGH* transgenic lines, all five lines bearing the *HSIII,VhGH* construct expressed high levels of *hGH-N* in the kidney (Fig. 7B). Additional sporadic low-level expression was detected in the livers and brains of several lines by RT-PCR (not shown). As judged by Northern blot signal intensity, the kidney expression

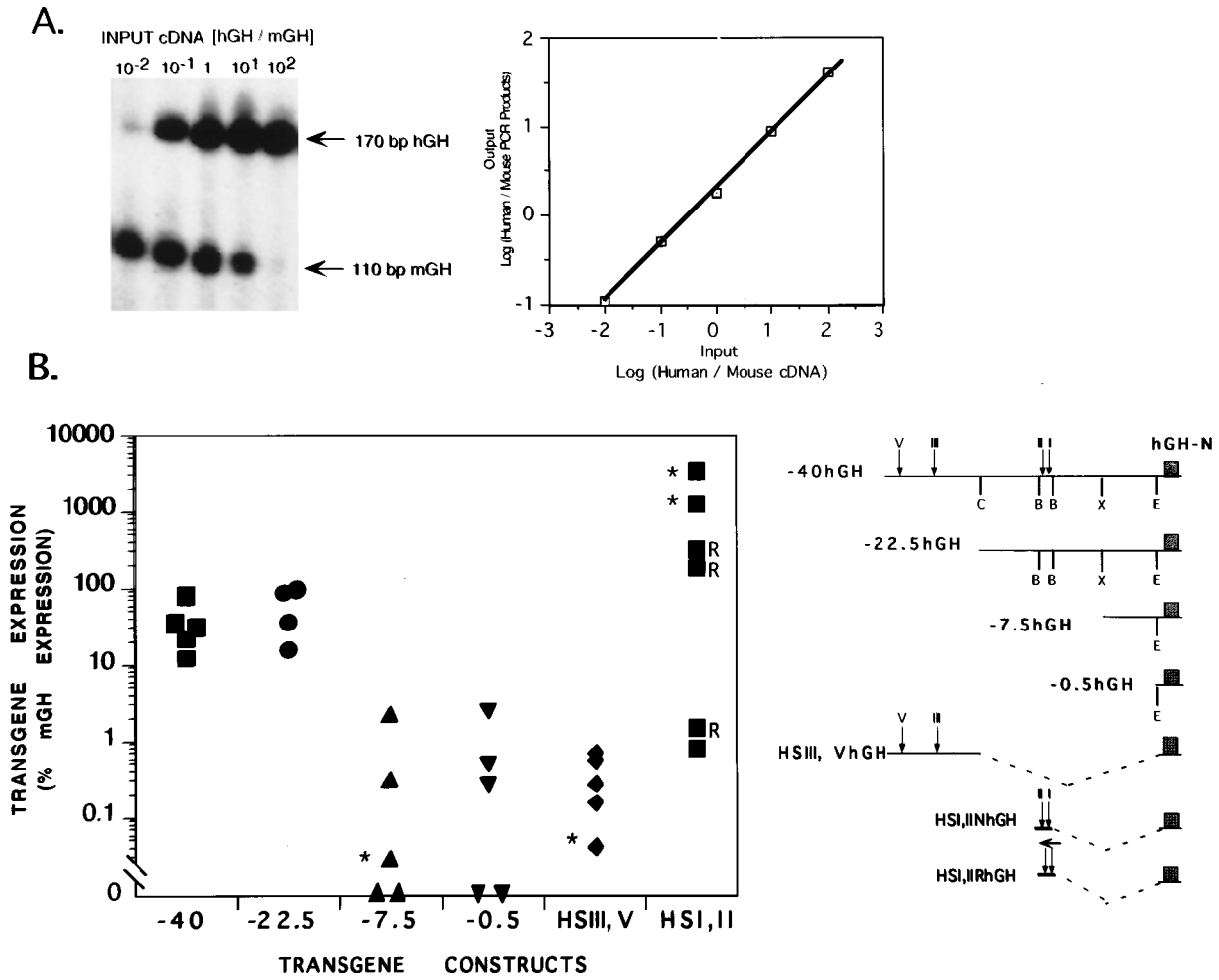


FIG. 6. Copy number dependence of transgene expression in the mouse pituitary. (A) Control amplifications of defined mixtures of *hGH* and *mGH* cDNAs. On the autoradiograph of the amplification (left), the ratios of the input *hGH* and *mGH* cDNAs are shown above the lanes, and the positions of the *hGH* and *mGH* cDNA amplification products are indicated at the right. The relationship of input ratio to output amplification products is shown in the graph to the right. Such standard curves were used to determine the transgene expression ratios displayed in panel B. (B) Expression of each transgene per copy relative to that of endogenous pituitary *mGH*. The level of pituitary *hGH-N* mRNA per transgene copy relative to that of a single copy of the endogenous *mGH* gene is plotted along the ordinate. Note that the values are displayed on a logarithmic scale. Each individual transgenic line is indicated by an independent datum point. A representation of the structure of each of the transgenes is shown at the right. Several datum points either exceed or are below the linear range of the assay and are marked (*). The orientation of the HS I,II region relative to the promoter is indicated if reversed (R).

of *HSIII,VhGH* was severalfold greater than that in the pituitary. When corrected for gel loading and transgene copy number, the renal expression levels varied by sixfold or less among the five lines studied (Fig. 7B).

Since hGH-N is secreted into the circulation even when expressed ectopically, it was possible to use the ratio of serum hGH levels to pituitary *hGH* mRNA levels as a sensitive indicator of ectopic expression irrespective of its tissue origin (Fig. 8). The average ratios in the five *−40hGH* lines ranged from 1.3 to 4.9. These values were taken as indicative of pituitary-specific transgene expression because no ectopic expression was detected (see above). In comparison with the *−40hGH* lines, three of the four *−22.5hGH* lines had high ratios (7.2, 11.3, and 18.3). This evidence of dysregulation in the *−22.5hGH* lines was consistent with the observation that at least one of these lines expressed trace levels of *hGH-N* ectopically in the spleen. More marked evidence for loss of tissue specificity and dysregulation was seen with *HSIII,VhGH*. All five lines had serum hGH/pituitary *hGH-N* RNA ratios that were at least 100-fold greater (range, 485 to 3,173) than in the *−40hGH* lines. This high ratio is consistent with the documented high-level ectopic expression in the *HSIII,VhGH* kidneys. An accurate calculation of the serum hGH/pituitary *hGH* mRNA ratio was not possible in the case of the HS I,II lines because of the very high levels of transgene expression. Evidence for ectopic expression was also detected for transgenic lines lacking HS; the ratios in all three *−7.5hGH* lines studied were significantly elevated. By this parameter, only the *−40hGH* lines demonstrated strict tissue-specific expression of the transgene.

Pituitary expression of *GH*, and hence somatic growth, is under multiple levels of control mediated primarily through hypothalamic signals. To determine whether the *hGH* transgenes had sufficient *cis* elements to maintain appropriate physiologic control, growth curves based on body weights were established for multiple lines containing *hGH* linked to one or both sets of HS (Fig. 9). Transgenic and nontransgenic littermates in all three *−40hGH* lines grew identically. This was also true of the *HSIII,VhGH* lines. In contrast, one of the *−22.5hGH* lines (110A) demonstrated clear gigantism among the transgenic pups. In the case of the *HSI,IIhGH* lines, the incidence of

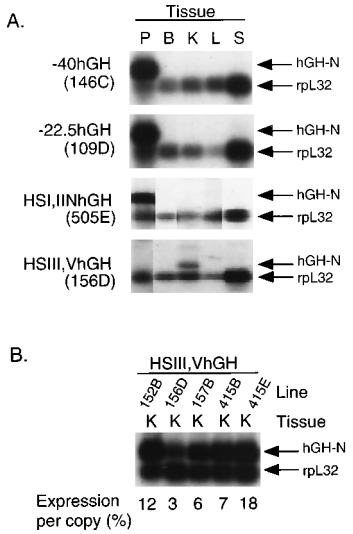


FIG. 7. Tissue specificity of *hGH-N* transgene expression. (A) Northern analyses of representative transgenic lines. The construct and specific line analyzed are indicated at the left, and the source of the RNA is indicated above each lane: pituitary (P), brain (B), kidney (K), liver (L), and spleen (S). Each Northern blot was sequentially probed with *hGH-N* cDNA and then mouse ribosomal protein L32 (rpL32) cDNA without stripping. (B) Each of five independent *HSIII,VhGH* lines expressed *hGH-N* in their kidneys (K) in a transgene copy number-dependent fashion. Northern analyses of kidneys from each of the five *HSIII,VhGH* lines are shown. The level of *hGH-N* expression is shown for each line below the corresponding lane as a percent of endogenous mouse ribosomal protein L32 mRNA.

gigantism was quite high. When oriented in the sense direction with respect to the *hGH* promoter (*HSI,IINhGH*), all four lines or founders that could be evaluated were giants. In addition, the three founders that died prior to assessment are likely to have died as a result of uncontrolled levels of hGH (8). Orientation of HS I and II in the antisense direction (*HSI,IINhGH*) resulted in gigantism, although with a lower incidence (one of three lines). These data suggested that sequences in addition to HS I and II are required for appropriate physiologic and tissue-specific regulation of *hGH* gene expression in the postnatal state. In the case of the HS I,II lines, direct juxtaposition of the HS I,II fragment to *hGH* may also contribute to transgene dysregulation.

## DISCUSSION

In this study, we have shown that the *hGH-N* gene along with as much as 7.5 kb of contiguous 5′-flanking sequences was highly sensitive to chromatin position effects in transgenic mice. Inclusion of 40 kb of contiguous 5′-flanking region resulted in reproducible, copy number-dependent, and pituitary-specific expression of *hGH-N* at levels that were comparable to those of endogenous *mGH*. The normal growth patterns in the five independent *−40hGH* lines combined with the lack of evidence for ectopic expression of the transgene, at both mRNA and protein levels, supported the possibility that the *−40hGH* transgene was under normal tissue-specific and physiologic regulation. These findings suggested that one or more elements necessary for appropriate expression of *hGH-N* in a native chromatin environment are located between 40 and 7.5 kb 5′ to the *hGH* cluster.

The region from 7.5 to 40 kb 5′ to the *hGH-N* cluster contained four DNase I HS in pituitary chromatin (Fig. 2). At least three of these HS (I, II, and III) were reestablished in pituitaries of mice transgenic for *−40hGH* (Fig. 3). The positioning, tissue specificity, and re-formation of these HS in transgenic mice suggested that they might contribute to the establishment of an active transcriptional domain in the pituitary and constitute components of the *hGH* LCR.

Deletion analysis of the *hGH-N* 5′-flanking region was carried out to assess the contribution of the two sets of HS to position independence and copy number dependence. Nine of nine lines containing HS I and II in their native positions (*−40hGH* and *−22.5hGH*) expressed *hGH-N* in their pituitaries in a reproducible manner and at levels that correlated with transgene copy numbers. Levels of expression per transgene copy in these lines were tightly grouped and averaged 47% of the endogenous *mGH* (Fig. 6B). The observed clustering of expression levels per transgene copy stands in marked contrast to the expression of the remaining transgene constructs. Truncation of the 5′-flanking region to a position 3′ of HS I eliminated the ability of the transgene to overcome position effects. Five of eleven such lines with transgene constructs, *−7.5hGH*, *−5.0hGH*, and *−0.5hGH*, failed to express *hGH-N* in the pituitary. The levels of expression in the remaining lines were low and varied widely, averaging 1.2% of endogenous *mGH* levels.

When tested in isolation, HS I and II appeared to have a potent enhancing effect on *hGH-N* expression. Direct linkage of the 1.6-kb *Bgl*II fragment containing HS I and II to the minimal *−0.5hGH* gene rendered expression of the resultant transgene site-of-integration independent. Six of six lines with transgene constructs *HSI,IINhGH* and *HSI,IIRhGH* expressed *hGH* in the pituitary (Fig. 5). The HS I,II segment boosted the mean expression level of the minimal *−0.5hGH* transgene by 1,000-fold. The growth curves and ectopic expression results indicate that the HS I,II segment alone, while mediating a potent enhancer activity, cannot fully establish normal patterns of *hGH* expression. Even when the HS I,II region was tested in its native context relative to *hGH-N* (*−22.5hGH*), there was evidence for a loss of tissue specificity as compared with *−40hGH*. This conclusion was suggested by assay of mRNA from multiple tissues on overexposed Northern blots and/or RT-PCR and was confirmed by the mild elevation of the serum hGH/pituitary *hGH* mRNA ratio (Fig. 8). Furthermore, gigantism was noted in one of the *−22.5hGH* lines studied, indicating continued susceptibility to chromatin position. The insufficiency of the HS I,II segment to fully constitute LCR function

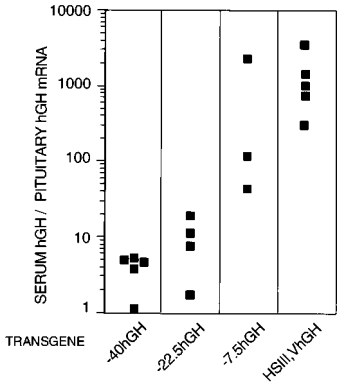


FIG. 8. Tissue specificity of *hGH* transgene expression as measured by the ratio of serum hGH to pituitary *hGH* mRNA. Multiple independent lines carrying each of the four indicated transgenes were assayed for specificity of hGH synthesis and secretion from the pituitary. The values were plotted, and the ordinate is logarithmic. High serum hGH/pituitary *hGH* mRNA ratios suggest ectopic expression of the *hGH* transgene.

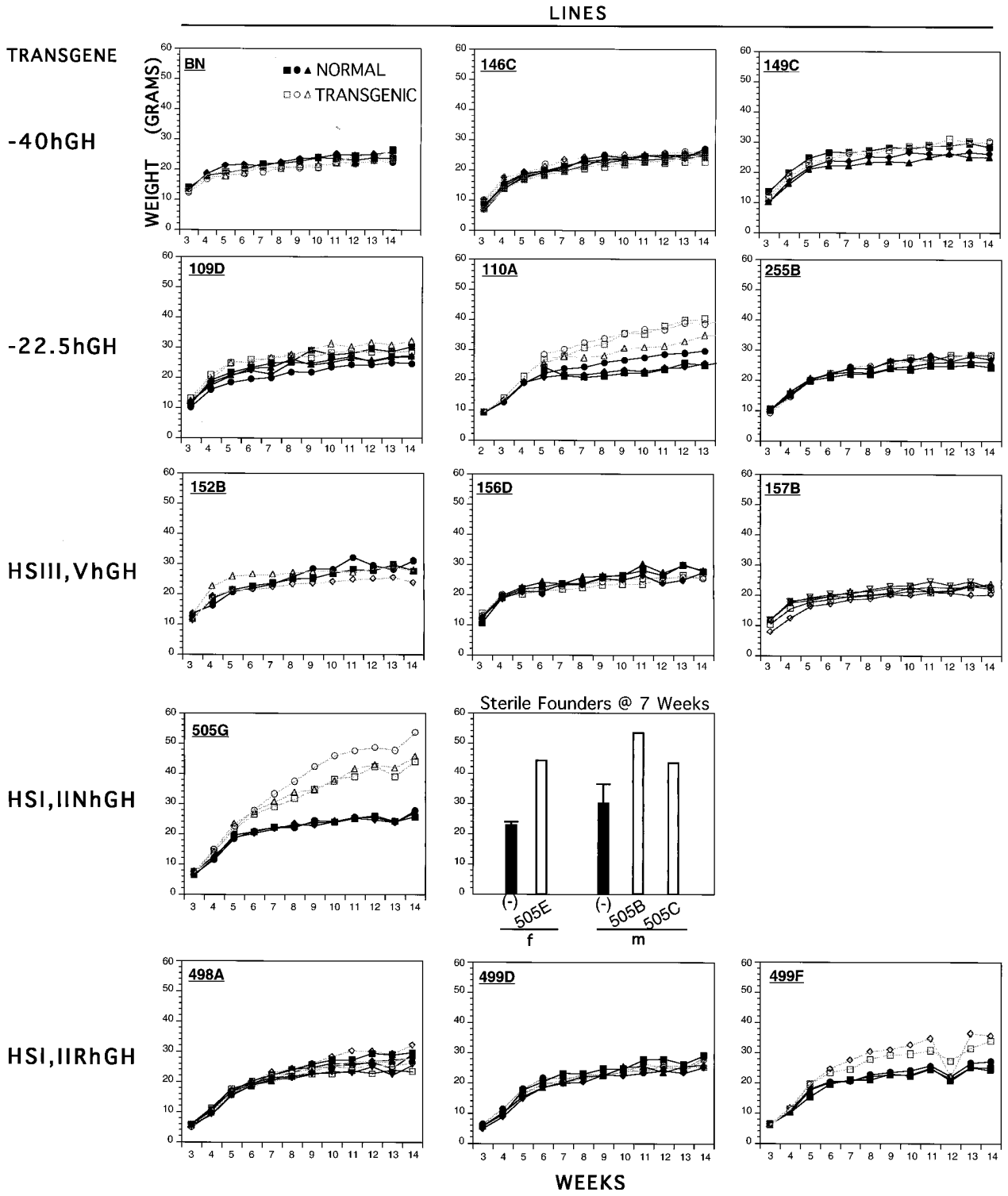


FIG. 9. Growth curves indicate that gigantism is limited to transgenic lines in which *hGH* is under the control of HS I and II. Body weights of transgenic pups (open symbols) and their wild-type littermates (filled symbols) were determined over 14 weeks. In three cases for which lines could not be established because of sterility of the founders (*HSI,IINhGH*), the weight of the founder at 7 weeks of age was determined and compared with the mean weight (± standard deviation) of normal mice at the same age (bar graph). All comparisons were sex matched, and all pups were female (f) unless otherwise noted. m, male.

was even more evident when tested in isolation (*HSI,IINhGH* and *HSI,IIRhGH*); pituitary *hGH* mRNA levels were extraordinarily high in at least four lines, and the level of expression per transgene copy spanned approximately 3 orders of magnitude (Fig. 5 and 6). In addition, the expression of serum hGH in these lines was unregulated and supraphysiologic, resulting in a high frequency of infertility, mortality, and gigantism. Whether this dysregulation reflected the deletion of critical

elements either 5′ or 3′ of the 1.6-kb *Bgl*II fragment containing the two HS or whether it reflected the direct juxtaposition of these elements to the *hGH* promoter was not established.

The DNA segment encompassing HS III and HS V also appeared to contribute to appropriate expression of the *hGH* transgene, although through a mechanism markedly different from that of the HS I,II enhancer function. Deletion of this region from the *−40hGH* gene, while leaving HS I,II intact (*−22.5hGH*), resulted in some degree of dysregulation of the transgene as noted above. Juxtaposition of the fragment containing HS III and V to the *−0.5hGH* gene (*HSIII,VhGH* lines) resulted in consistent pituitary expression (five of five lines; Fig. 5). This ability to establish position independence was further supported by the unexpected finding that the ectopic expression of *HSIII,VhGH* in the kidney was also reproducible and transgene copy number dependent. While the reasons for the ectopic kidney expression were not determined, this finding of copy number dependence suggested that the HS III,V segment could establish its own stable chromatin environment irrespective of transgene insertion site.

A major difference between the HS I,II and HS III,V segments when studied in isolation is the significantly higher levels of expression in the former lines. The overall levels of *hGH* expression per transgene copy in the pituitaries of *HSIII,VhGH* transgenic mice were less than 1% of the levels in the *−22.5hGH* lines and less than 0.1% of the levels in the *HSI, IINhGH* lines (Fig. 6B). This marked contrast in *hGH* expression levels corroborated the suggestion made above that the HS I,II region contained an enhancer-like activity not present in the HS III,V region. The ability of the HS III,V region to overcome chromatin position effects suggested that it may contain a function demarcating a chromatin domain, providing an accessible chromatin environment regardless of the site of integration. It might also function by blocking the entry of influences from neighboring chromatin elements native to the insertion site. In this way, the deletion of the HS III,V region might have caused *−22.5hGH* to be more susceptible to position effects. Thus, the HS I,II region appeared to act like a major enhancer, while the sites common to the pituitary and placenta (HS III and V) act to open chromatin and/or act as a boundary element.

The unexpected ability of *HSIII,VhGH* to direct *hGH* expression to the kidney in a reproducible manner (Fig. 7B) may be worth further note. The levels of renal expression determined by Northern blotting were substantial. The high-level renal expression might have been facilitated by interaction between the HS and/or the *hGH-N* promoter with the transcription factor pit-1, which is detectable in the kidney (59). Such an interaction must be precluded by the native configuration of the 5′-flanking region of *hGH* because renal expression was not evident when HS III and HS V were in their native locations (*−40hGH*). Deletion of sequences between kb −22.5 and −0.5 may dysregulate the tissue specificity of this gene in a unique manner. For example, a kidney-specific silencer may have been removed. Furthermore, it is possible that elements within the HS III,V fragment may be shared by kidney-specific genes. Since LCR elements that mediate control of genes in the kidney are not presently available, use of constructs containing HS III and V might be useful in targeting transgenes to the kidney.

The tightest combination of tissue specificity and copy number dependence was noted for the *−40hGH* transgene insert containing all four HS in their native positions. This observation is consistent with the conclusion that both HS subsegments contributed important and nonoverlapping functions. The *hGH* cluster and its associated LCR can be compared in this regard with the human β-globin gene locus and its LCR. The β-globin gene cluster and *hGH* gene clusters both span approximately 50 kb, contain five active genes, and are expressed at very high levels with tightly controlled tissue specificity. The LCRs of both loci contain four dispersed HS located quite distally 5′ from their respective gene clusters. No single HS replaces the full set in establishing reproducible, copy number-dependent transgene expression both for the β-globin gene (24, 25, 53) and for *hGH* (this report). The β-globin gene cluster is solely expressed in erythroid tissues, whereas the *hGH* cluster is expressed in two discrete tissues, the pituitary and placenta. The site-of-integration dependence of the *15kbhCS-A* transgene mimics that of the *−0.5hGH*. Whether juxtaposition of LCR elements will overcome the *hCS-A* position effect can now be tested. If this is the case, the mutually exclusive pituitary and placental expression of the genes in this cluster may reflect competition for a common LCR or utilization of different subsets or components of this region. HS IV, unique to placental chromatin, may be important in this regard. The proposed mechanisms of LCR competition and LCR component (HS) specificity that have been invoked to explain the developmentally controlled switching in the β-globin gene cluster (20) can now be applied to understanding the dual tissue specificity within the *hGH* cluster.

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## ADDENDUM IN PROOF

We have recently determined that HS III to V of the *hGH* LCR are located in the adjacent muscle-specific sodium channel α-subunit gene (*SCN4A* locus) (I. M. Bennani-Baiti, B. K. Jones, S. A. Liebhaber, and N. E. Cooke, Genomics **29:**647–652, 1995).

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